ALCHEMY Jurnal Penelitian Kimia

Official Website: https://jurnal.uns.ac.id/alchemy

ISSN 1412-4092
EISSN 2443-4183

Isolation and Characterization of Bioactive Compounds from Mangrove Sediment Actinomycetes with Antibacterial Activity Against *Staphylococcus aureus*

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DOI: 10.20961/alchemy.22.2.112221.381-396

Received 12 January 2026, Revised 4 May 2026, Accepted 7 May 2026, Published 30 September 2026

Keywords:

actinomycetes; antibacterial; chitin; mangrove sediment.

ABSTRACT. Antibiotic resistance has intensified the search for new bioactive compounds. This study aimed to isolate actinomycetes from mangrove sediments, evaluate their antibacterial acvity by microdilution, and characterize the active compounds. Sediment samples were collected from three sites in the Sriminosari Mangrove Forest Area, East Lampung. Actinomycetes were isolated on colloidal chitin agar and purified. A selected isolate was cultivated on 50 g of solid shrimp-shell medium under static conditions for 14 days and extracted with ethyl acetate. The crude extract was screened against *Staphylococcus aureus* in 96-well microtiter plates. The most promising isolate was scaled up using 200 g shrimp shells, and its crude extract was fractionated by column chromatography and assayed against *S. aureus*. The active fraction was characterized by Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and Fourier-transform spectroscopy (FTIR). Seven actinomycete strains were obtained, belonging to *Streptomyces* and *Planomonospora*. Strain ISM 7 showed the strongest inhibitory activity against *S. aureus*. The active fraction ISM7K1F3 contained alkaloids with a morpholine basic structure, as indicated by functional groups including O–H, alkane C–H stretching, C=O, C–N, alkane C–H bending, and C–O. These findings suggest that mangrove-derived actinomycetes, particularly strain ISM 7, are promising sources of antibacterial alkaloids.

INTRODUCTION

The phenomenon of the emergence of pathogens that are resistant to antibiotics causes human health to be increasingly threatened. *S. aureus* is one of the main pathogenic bacteria that can cause many infectious diseases both in hospitals and the community environment. *S. aureus* bacteria are classified as part of the ESKAPE group *(Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter),* which represent a significant category of bacteria implicated in infections characterised by multidrug resistance (de Oliveira *et al.*, 2020). *S. aureus* occupies a distinctive position among other bacterial species due to its relatively high virulence and high degree of plasticity, which enables it to adapt to a variety of environmental conditions. Strains of *S. aureus* have developed mechanisms of resistance to almost all antimicrobial drugs used in medicine, including those most commonly used to treat Gram-positive infections, such as beta-lactams, glycopeptides, and oxazolidinones (Mlynarczyk-Bonikowska *et al.*, 2022). This event encouraged researchers to explore new bioactive compounds.

The development and discovery of new secondary metabolite compounds from natural materials is important to explore in order to find new antibiotic compounds to overcome these resistant pathogenic bacteria. One of the natural ecosystems that is still underexplored is mangroves. A literature review reveals that mangroves are unique ecosystems with high salinity, low pH values and periodic tidal submersion. The environmental complexity of the mangrove ecosystem is what causes the diversity of microorganisms including actinomycetes (Guo *et al.*, 2015; He *et al.*, 2019). Mangrove sediments also support and help the formation of cooperative aerobic and anaerobic microenvironments and the influence of extreme environmental conditions can increase the diversity of

Cite this as: Bahri, S., Setiawan, A., Setiawan, W. A., Ambarwati, Y., and Prasetya, I. (2026). Isolation and Characterization of Bioactive Compounds from Mangrove Sediment Actinomycetes with Antibacterial Activity Against *Staphylococcus aureus*. *ALCHEMY Jurnal Penelitian Kimia*, 22(2), 381-396. doi: https://dx.doi.org/10.20961/alchemy.22.2.112221.381-396.

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microorganism communities in sediments (Law *et al.*, 2019). Types of microorganisms originating from mangrove sediments, such as actinomycetes, can produce many new bioactive compounds. Several bioactive compounds are produced, such as alkaloids, terpenoids, steroids, and polyketides which have various activities, including antibacterial, enzyme inhibitor, anti-inflammatory, antioxidant, antiviral, and cytotoxic activities (Cai *et al.*, 2021). Mangroves have been considered as one of the rich resources for isolating actinomycetes. It has also been reported that there has been an increase in the number of *Streptomyces* strains originating from mangroves that show strong bioactivity, such as antimicrobial, anticancer, and antioxidant (Mangamuri *et al.*, 2016; Tan *et al.*, 2018).

The mangrove ecosystem in Indonesia is very extensive, but research on antibacterial bioactive compounds originating from mangrove sediments has not been widely explored, especially mangroves in Lampung province. This research is focused on the discovery of alkaloid compounds as antibacterial compounds originating from actinomycetes. Alkaloids are known to have high potential due to the diversity of their bioactivity and are an inspiration for the development of antibacterial drugs (Ryu *et al.*, 2019; Shaala *et al.*, 2020; Wang *et al.*, 2021). Information regarding antibiotic compounds produced by actinomycetes cultured on solid shrimp shell media is still limited. Therefore, this research aims to isolate actinomycetes from mangrove sediments, obtain cultured extracts, purify, and characterize cultured extracts that show potential antibacterial activity against *S. aureus*.

RESEARCH METHODS

Isolation of Mangrove Sediment Actinomycetes

Mangrove sediment samples were collected at approximately 10 – 20 m intervals in the Sriminosari Mangrove Forest Area in East Lampung. Samples were taken using a sterile spoon at a depth of ±10 cm, put in a plastic clip, and labeled. Samples were stored in a cool box at 4 ºC (Li *et al.*, 2009). A total of 1 g of sample was transferred into a test tube containing 9 mL of sterile artificial seawater and subjected to serial dilution. A 100 µL aliquot was spread on colloidal chitin agar supplemented with 50 µg/mL ciprofloxacin and incubated at room temperature for 14 days. The colloidal chitin agar medium (g L^{-1}) contained 1.0 g colloidal chitin, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, and 15 g agar. Actinomycetes isolates were subsequently cultured on agar medium containing 1% colloidal chitin and sterile artificial seawater. The artificial seawater composition (g L^{-1}) was 27.0 g NaCl, 5.6 g $MgCl_2 \cdot 6H_2O$, 1.5 g $CaCl_2 \cdot 2H_2O$, 1.0 g KNO_3, 0.07 g K_2HPO_4, 6.6 g $MgSO_4 \cdot 7H_2O$, and 0.04 g $NaHCO_3$ (Setiawan *et al.*, 2022). Most chemicals and reagents used in this study were purchased from Merck (Darmstadt, Germany), unless otherwise stated.

Microscopic Identification of Mangrove Sediment Actinomycetes

Single isolates were identified microscopically using the coverslip slide culture method. A coverslip measuring 22 × 22 mm was inserted at a 45° angle in the middle of the colloidal chitin agar medium, the colonies were streaked close to the coverslip, and the cultures were incubated for 4 to 7 days. The coverslip was removed slowly using sterile tweezers and placed on a clean glass slide, then observed at 400× magnification using an Axio Lab.A1 microscope (Carl Zeiss Microscopy GmbH, Germany) (Setiawan *et al.*, 2021).

Cultivation

Actinomycetes isolates were cultured on solid shrimp shell media using the solid-state fermentation method which refers to the method from Setiawan *et al.* (2021). Actinomycetes isolates were grown in liquid inoculum medium containing 1% chitin colloid in 20 mL of sterile artificial seawater and incubated at room temperature for 7 days. The grown actinomycetes inoculum was transferred into a 500 mL Erlenmeyer flask containing 50 g of sterile shrimp shells and then incubated at room temperature for 14 days. Actinomycetes biomass was macerated using ethyl acetate (EtOAc) solvent for 1 day. The filtrate was filtered and concentrated using a Büchi rotary evaporator (Büchi Labortechnik AG, Switzerland). The concentrated crude extract was then weighed.

Antibacterial Activity Screening Against *S. aureus*

The isolate of the pathogenic bacterium S. aureus was obtained from the UPT-LTSIT, University of Lampung, and originated from Abdul Muluk Hospital, Bandar Lampung. The bacteria had previously been reported to be resistant to clindamycin, ciprofloxacin, erythromycin, lincomycin, and amoxicillin (Lutfiah *et al.*, 2021). Antibacterial screening followed the Clinical and Laboratory Standards Institute (CLSI) approach using 96-well microtiter plates. The bacterial suspension was prepared in 5 mL of Tryptic Soy Broth (TSB), adjusted to 0.5 McFarland (OD630= 0.08 – 0.1), and diluted 1:100 (10^6 CFU/mL). The crude extract was prepared at 2 mg/mL;

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chloramphenicol (2 mg/mL) was used as the positive control, 12.5% methanol as the negative control, TSB as the growth control, and uninoculated medium as the sterile control. Each well contained 145 µL TSB, 50 µL sample extract, and 25 µL bacterial suspension. The microtiter plates were incubated at 37 °C for 18 h. Absorbance was measured using a Hospitex microplate reader. Then, 30 µL of 0.02% resazurin was added, followed by incubation for 2 – 4 h to observe the color change (Elshikh *et al.*, 2016).

Thin Layer Chromatography Separation (TLC)

Crude extracts (crude) of all actinomycetes isolates that had been screened were then separated into compound components via thin-layer chromatography using F254 silica gel plates. The crude extract (crude) was smeared a little on the plate and eluted under saturated conditions using several eluents and in various ratios, such as 100% ethyl acetate, 100% n-hexane, and n-Hexane:ethyl acetate (7:3). The plates were visualized under 254 nm UV light and then reacted with several reagents, including cerium(IV) sulfate and specific Dragendorff reagent (solution 1: 1.7 g bismuth nitrate with 80 mL distilled water and 20 mL glacial acetic acid; solution 2: solution KI (50% w/v, 100 mL) with glacial acetic acid) (Setiawan *et al.*, 2022).

Cultivation (Scale Up) and Extraction

Isolates that are known to have the greatest antibacterial activity (potential isolates) are cultivated on a large scale. Actinomycetes isolates were grown in liquid inoculum media, each containing 1% chitin colloid, in 50 mL of sterile artificial seawater and incubated at room temperature for 7 days. The composition of artificial seawater (g L^{-1}) consisted of 27.0 g NaCl, 5.6 g $MgCl_2 \cdot 6H_2O$, 1.5 g $CaCl_2 \cdot 2H_2O$, 1.0 g KNO_3, 0.07 g K_2HPO_4, 6.6 g $MgSO_4 \cdot 7H_2O$, and 0.04 g $NaHCO_3$. The actinomycetes inoculum that was 7 days old was transferred into a 2.5 L dark bottle containing 200 g of sterile shrimp shell substrate, and 5 repetitions were made, then incubated at room temperature for 14 days. The actinomycetes biomass that had grown after 14 days of incubation was macerated using ethyl acetate (EtOAc) solvent for 1 day. The filtrate was filtered and concentrated using a Büchi rotary evaporator (Büchi Labortechnik AG, Switzerland). The crude extract (crude), which had become concentrated, was then weighed (Setiawan *et al.*, 2021).

Column chromatography fractionation

Crude extracts (crude) of superior isolates that have been obtained as a result of large-scale cultivation are separated from their compound components using thin-layer chromatography (TLC) using several types and ratios of eluents to determine the type of solvent and the appropriate ratio for column chromatography fractionation. Fractionation via open column chromatography using silica gel (SiO_2) as a hydrated stationary phase using n-hexane solvent. Elution begins with 100% n-hexane solvent, n-Hexane: ethyl acetate (9:1), n-Hexane: ethyl acetate (7:3), and ends with methanol. The fractionation results are then subjected to TLC using the appropriate eluent. Fractions that have the same Rf value can be combined. Next, each fraction was tested for its antibacterial activity against *S. aureus* to determine the active fraction. The fraction with the highest activity was purified until one spot appeared on the TLC which was eluted using several variations of eluent (Sudding *et al.*, 2021).

Compound characterization

The active fraction of the purified sample was characterized using a Cary 630 Fourier Transform Infrared Spectrometer (Agilent Technologies, USA). Positive-mode LC-MS/MS analysis was performed after dissolving the active fraction in methanol. The LC-MS/MS system comprised an ACQUITY UPLC® H-Class System, an ACQUITY UPLC® HSS C18 column (1.8 µm, 2.1 × 100 mm), and a Xevo G2-S QTof Mass Spectrometer (Waters, Beverly, MA, USA).

RESULTS AND DISCUSSION

Material Sampling

Mangrove sediment samples have been taken. Samples were taken from three points with a radius of 90 meters in the Sriminosari Mangrove Forest area, East Lampung, including point A (5°18'59.5"S 105°49'20.0"E), point B (5°18'58.7"S 105°49'20.2" E), and point C (5°19'01.6"S 105°49'19.8"E). Based on observations of the surrounding area, it is known that the Sriminosari mangrove forest area in East Lampung is located near ponds and rice fields. This condition is likely to affect the sustainability of the mangrove ecosystem, however, the mangrove forest area is also very close to the sea area where tides frequently occur, so that the area remains

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maintained and protected from pollution from population activities. Mangrove sediment samples taken from each point have different colors and textures, as shown in Table 1. Various environmental factors, including temperature, weather and pH, may be the cause of these differences (Guo *et al.*, 2015; He *et al.*, 2019). Mangrove sediment is a source of carbon and organic substances that are characterized by a black-brown color. Sandy textures have a lower concentration of organic substances than non-sandy textures. Mangrove sediment samples A, B, and C are brownish in color, but samples A and C contain more sand than sample B, so organic nutrients are likely to be lower than in sample B. According to Bissett *et al.* (2007) organic nutrients in mangrove sediments can influence the level of microbial diversity.

Table 1. The texture of the sediment samples from three different points in the Sriminosari Mangrove Forest Area, East Lampung.

Point	Sampling Area	Mangrove Sediment Samples	Information
A			The sample is brownish and sandy in color.
B			The sample is brownish and slightly sandy.
C			The sample is blackish brown and sandy.

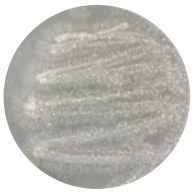
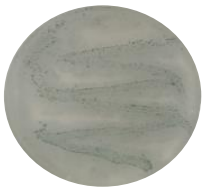
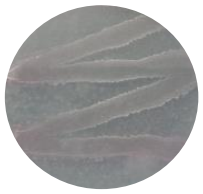
Isolation of actinomycetes from mangrove sediments

Isolated actinomycetes were grown on selective colloidal chitin media containing nitrogen and carbon nutrients (Lacombe-Harvey *et al.*, 2018). Colloidal chitin is a type of polysaccharide that is difficult to degrade, while actinomycetes have the ability as decomposers, so they can grow on colloidal chitin agar media (Golinska *et al.*, 2015; Ranjani *et al.*, 2016). The isolation results were observed macroscopically after 14 days of incubation, because actinomycetes generally require a longer incubation period to form visible colonies (Gokulan *et al.*, 2014). The results of the observations showed that the growing colonies were still mixed, so microbial isolation was carried out continuously until pure cultures were obtained. The macroscopic observation of pure cultures grown includes the shape of the colony, the colony's edge, and the aerial mycelium. There are 7 pure cultures isolated from Sriminosari mangrove sediment samples, which can be seen in Table 2.

Macroscopic observation of the actinomycetes isolates revealed significant variation in characteristics such as shape and colour. The diversity of pure cultures is dominated by sample B, which contains little sand, in contrast to samples A and C, whose mangrove sediment samples contain more sand, so that fewer pure cultures are obtained. This is in accordance with literature which shows that the organic nutrient content in mangrove sediments can influence the level of microbial diversity (Bissett *et al.*, 2007; Sharqawy *et al.*, 2010).

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Table 2. Macroscopic identification of mangrove sediment actinomycetes isolates.

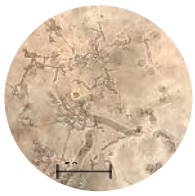
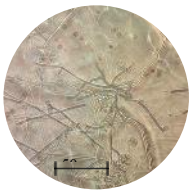
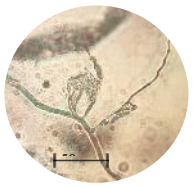
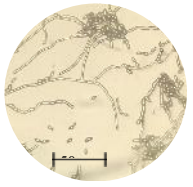
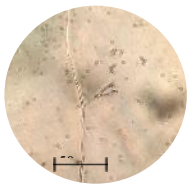
Sample Code	Isolate Code	Picture	Colony Form	Edge of the Colony	Aerial Mycelium
B	ISM 1		Round	Stringy	White
B	ISM 2		Round	Threaded and serrated	Brownish red
C	ISM 3		Round	Threaded	Blue
B	ISM 4		Round	Threaded	Beige
A	ISM 5		Round	Threaded	Grayish green
B	ISM 6		Round	Threaded	Grayish brown
C	ISM 7		Round	Jagged	White pink

Microscopic Identification of Mangrove Sediment Actinomycetes Isolates

Pure cultures were cultivated on colloidal chitin agar media, which were then subjected to a five-day incubation period under static conditions. Thereafter, identification was conducted microscopically using an Axio Zeiss A1 microscope at 400× magnification. The results of the observations are shown in Table 3, actinomycetes isolates are dominated by long-chain spore forms, which are indicated by the genus *Streptomyces*, namely isolates ISM1, ISM2, ISM 3, ISM5, ISM 6, and ISM7, while ISM 4 is thought to be indicated by the genus Planomonospora, which has leaf-like spore forms (Li *et al.*, 2016; Subramani and Sipkema, 2019). Spore diversity is also relatively varied, such as straight, rectiflexible, flexuous, monospore, and spiral chain (Li *et al.*, 2016; Risandiansyah and Yahya, 2021).

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Table 3. Microscopic characterization of actinomycetes isolates from mangrove sediments at 400x magnification.

Isolate	Picture	Spore Morphology	Genus Indication	Reference
ISM 1		Straight	*Streptomyces*	(Li *et al.*, 2016)
ISM 2		Spiral	*Streptomyces*	(Risandiansyah and Yahya, 2021)
ISM 3		Straight	*Streptomyces*	(Risandiansyah and Yahya, 2021)
ISM 4		Monospore	Planomonospora	(Messaoudi *et al.*, 2020)
ISM 5		Rectiflexibiles	*Streptomyces*	(Li *et al.*, 2016)
ISM 6		Rectiflexibiles	*Streptomyces*	(Risandiansyah and Yahya, 2021)
ISM 7		Flexous	*Streptomyces*	(Risandiansyah and Yahya, 2021)

Heul *et al.* (2018) stated that most of the *Streptomyces* genus is the genus that produces the most secondary metabolite compounds, especially antibacterial compounds. Several studies also report that bioactive compounds known to have antibacterial activity have been isolated from the genus, while the rare actinomycetes or non-*Streptomyces* actinomycetes is also known to produce antibacterial secondary metabolite compounds (Messaoudi *et al.*, 2020). It is evident from the findings of research and literature studies concerning the potential bioactive compounds that can be produced by the *Streptomyces* and *Planomonospora* genera that all actinomycetes isolates have the potential to be cultivated.

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Antibacterial Activity Screening Against *S. aureus*

Cultivation on 50 g of shrimp shell media has produced crude extracts, namely ISM 1 (30 mg), ISM 2 (44 mg), ISM 3 (30 mg), ISM 4 (32 mg), ISM 5 (46 mg), ISM 6 (31 mg), and ISM 7 (50 mg). A total of 50 µL samples of 2 mg/mL crude extract were screened for antibacterial activity against *S. aureus* in order to determine the superior isolate that exhibited the highest degree of antibacterial activity. The results of the antibacterial screening of the crude extract (crude) can be observed in Figure 1, which demonstrates the growth of *S. aureus* following the addition of the crude extract sample.

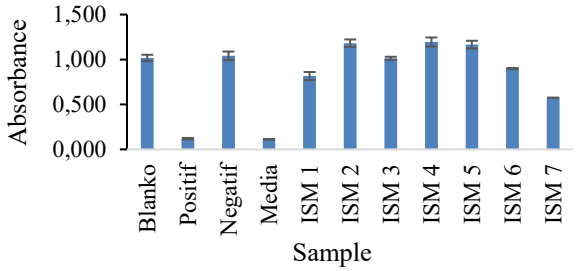


Figure 1. Growth of *S. aureus* after adding crude extract (crude).

As demonstrated in Figure 1, the inhibitory power of the crude extract of each isolate at a concentration of 454 µg/mL on bacterial growth was observed after 18 hours of incubation. The measurement of this inhibitory power was conducted at an absorption wavelength of 630 nm. This wavelength is a red absorption which indicates bacterial growth which is characterized by the reduction of resazurin (blue) to resofurin (pink). The blank or growth control indicates that the test bacteria used are still alive and able to grow, marked in pink after staining with resazurin, the positive control of chloramphenicol shows the inhibitory power of bacterial growth which is shown in blue, the 12.5% MeOH solvent control shows its absence. inhibitory effect on bacterial growth, and control media showed no contamination during the test (sterile). The growth curve of *S. aureus* shows the antibacterial activity of each crude extract from different isolates. Isolates ISM 1, ISM 6, and ISM 7 exhibited higher inhibitory activity, as indicated by lower bacterial growth relative to the growth control and closer to the positive control. Strain ISM 3 has very low inhibitory power, which is indicated by high bacterial growth, while strains ISM 2, ISM 4, and ISM 5 do not have inhibitory power, which is indicated by high bacterial growth (above the blanko results). Overall, ISM 7 crude extract was proven to have the highest inhibitory power against the growth of *S. aureus* bacteria. The differences in the ability to inhibit bacterial growth from each isolate may be caused by various factors, such as differences in compound components and the content of tertiary N groups (alkaloid components) in the samples. Wang *et al.* (2021) reported that several alkaloid compounds have antibacterial activity, so differences in the intensity of the alkaloid content in samples may also influence the amount of antibacterial activity produced.

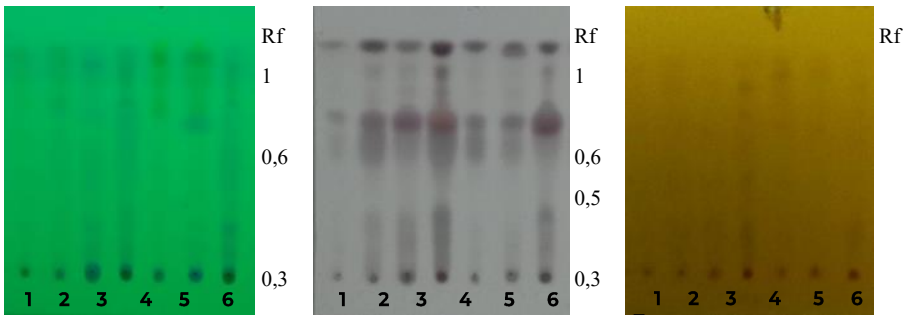
Thin Layer Chromatography Separation

The crude extract (crude) of actinomycetes isolates was subjected to thin-layer chromatography (TLC) in order to identify its compound components, as illustrated in Figure 2. The stationary phase of the experiment was a silica gel TLC plate, and the eluent was a mixture of n-Hexane:ethyl acetate in a ratio of 7:3. The TLC plate that had been spotted and eluted was observed under 254 nm UV light. The observation results in Figure 2a show that strains ISM 1, ISM 3, ISM 4, and ISM 7 produced elongated and very thin spots across Rf values of 0 – 1, while strains ISM 1, ISM 5, and ISM 6 showed spots at Rf 0 and elongated patterns from Rf 0.6 – 1. These elongated spots suggest the presence of complex mixtures rather than single compounds. The appearance of spots under UV light indicates the presence of UV-active compounds, although not all UV-active compounds necessarily contain conjugated C=C double bonds (Astuti *et al.*, 2021).

The TLC plate was then reacted with cerium(IV) sulfate reagent and 3 spots were obtained at different Rf values. Overall, each isolate sample showed almost the same compound separation pattern. The spots that appear are brownish red which indicates the presence of general organic compounds (Susilo and Suciati, 2016). Some of the spots formed were not detected when observed under 254 nm UV light, namely the middle spot at an Rf value of 0.5 – 0.6, which indicated that there were no conjugated C double bonds in the compound. Differences in spot thickness from each extract also indicate differences in the abundance of organic compounds from each strain, in

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Figure 2b. There are 4 types of strains with thicker spots, namely strains ISM 2, ISM 3, ISM 4, and ISM 7. Detection of tertiary N groups of alkaloid compounds in samples using a specific Dragendorff reagent which gives an orange color as a positive indication (Setiawan *et al.*, 2022). Observation results in Figure 2c. shows that the orange stain detected is only at the bottom at an Rf value of 0 with quite different concentrations for all strains. This shows that the abundance of tertiary N groups in the alkaloids of each strain is quite varied. The difference in tertiary N content in these samples may also influence the differences in antibacterial activity produced by each isolate, such as strains ISM 2, ISM 4, and ISM 5 which are known to have no antibacterial activity while the TLC results show the presence of alkaloid components. This shows that not all types of alkaloids have antibacterial activity (Wang *et al.*, 2020).



(a) (b) (c)

Figure 2. The TLC of crude extract with n-Hexane:ethyl acetate eluent (7:3): (a) UV at 254 nm, (b) Cerium(IV) sulfate, and (c) Dragendorff.

Cultivation and Extraction (Scale Up)

Cultivation using the solid-state fermentation method has been known to be effective in producing secondary metabolite compounds (Soccol *et al.*, 2017; Ooi *et al.*, 2021; Setiawan *et al.*, 2021, 2022). The use of shrimp shell waste was chosen as a substrate because of its high abundance in the seafood industry. The nutrients contained, such as protein and minerals, can be used to grow actinomycetes (Yazid *et al.*, 2017; Setiawan *et al.*, 2021). The superior isolate ISM 7, which is known to have the highest antibacterial activity, was then cultivated with 200 g of shrimp shells in 5 repetitions. The results of large-scale cultivation observations of the ISM 7 strain isolate are shown in Figure 3.

The ISM 7 strain isolate was observed to produce pink pigment and white spotted filaments after growing for 7 days on liquid colloidal chitin inoculum, and after growing for 14 days on shrimp shell media for 14 days it produced white spots that covered almost the entire surface of the substrate. Gokulan *et al.* (2014) stated that actinomycetes are in the stationary phase during the 14 day incubation period. The growth of the ISM 7 strain isolate on shrimp shell substrate shows that the isolate is able to degrade the substrate to be used as food, this is in accordance with the statement of Ranjani *et al.* (2016) that actinomycetes are capable of degrading all kinds of organic materials (they are decomposers).



(a) (b)

Figure 3. Growth of actinomycetes strain ISM 7: (a) liquid chitin colloid medium for 7 days and (b) solid shrimp shell fermentation medium for 14 days.

The actinomycetes isolate was then extracted using the maceration or immersion method using ethyl acetate (EtOAc) solvent. Maceration was chosen because it is easier and more effective to obtain secondary metabolite compounds from actinomycetes that grow on the surface of the shrimp shell substrate. Maceration was carried out for 1 day and remaceration was carried out to extract extracts that may have remained in the substrate container (Ćujić *et al.*, 2016; Verâne *et al.*, 2020; Setiawan *et al.*, 2021; Popovici *et al.*, 2022). The results of the maceration

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were concentrated using a Büchi rotary evaporator (Büchi Labortechnik AG, Switzerland) and a crude extract of 1.2 g was obtained. The crude extract is then purified via column chromatography.

Column Chromatography Fractionation

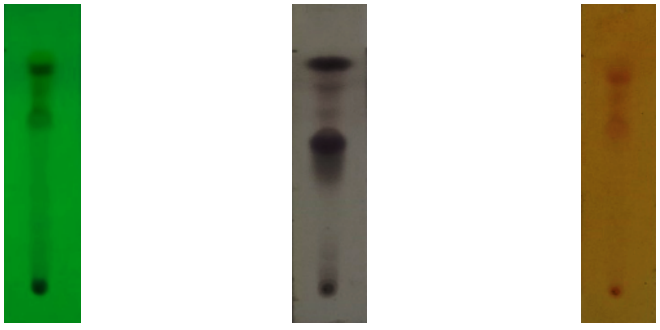
The purification process is achieved through column chromatography, which involves the separation of compounds based on their polarity levels as indicated by the results obtained from thin-layer chromatography. The crude extract obtained is then subjected to a second TLC separation, the purpose of which is to ascertain the compound pattern and determine the type and ratio of eluent that is suitable for use in column chromatography. Based on the TLC results of the ISM 7 sample in Figure 4, using the eluent n-Hexane:ethyl acetate (9:1), it can be seen that the separation pattern of compounds under 254 nm UV light at Rf 0.43, 0.06, and 0 indicated the presence of a conjugated C double bond (Astuti *et al.*, 2021). The next visualization used the cerium(IV) sulfate reagent, and a brownish-red spot was formed at Rf 0.9, 0.3, 0.23, and 0, which indicates the presence of organic compounds (Susilo and Suciati, 2016). Visualization using Dragendorff's reagent resulted in the formation of orange spots at Rf 0.3 and 0, which indicated the presence of tertiary N groups (Setiawan *et al.*, 2022). The eluent ratio n-Hexane:ethyl acetate (9:1) was then used to separate the Rf 0.9 spot (top fraction).



(a) (b) (c)

Figure 4. TLC of ISM 7 crude extract with n-Hexane:ethyl acetate (9:1) as eluent (a) UV 254 nm; (b) Cerium(IV) sulfate; and (c) Dragendorff.

The next eluent was determined using a ratio of n-Hexane:ethyl acetate (7:3) as shown in Figure 5. That is, there were visible spots under 254 nm UV light at Rf 0.93, 0.73, and 0. After being reacted with the cerium(IV) sulfate reagent, significant compound separation was seen at Rf 0.96, 0.6, and 0. Meanwhile, after being reacted with Dragendorff's reagent, the spots were at Rf 0.93, 0.73, and 0 which was orange. This eluent ratio can be used to separate compounds at Rf 0.6 (middle fraction) before finishing with methanol to separate the bottom fraction.



(a) (b) (c)

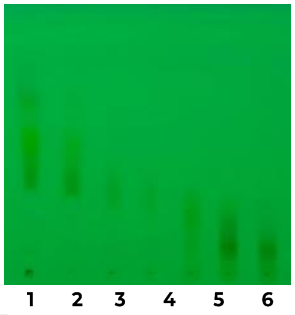
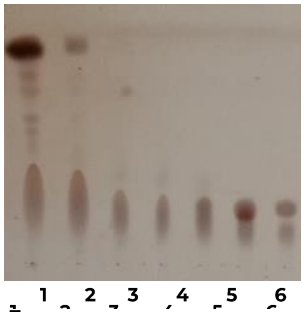
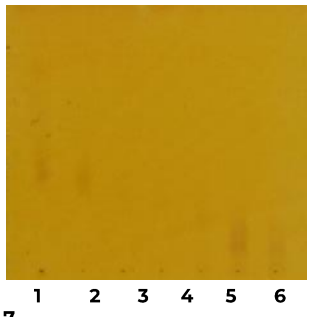
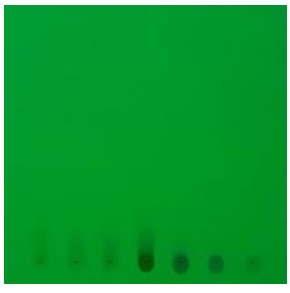
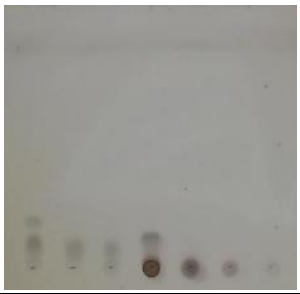
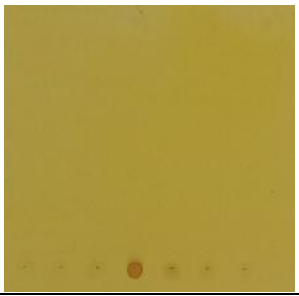
Figure 5. TLC of ISM 7 crude extract with n-Hexane:ethyl acetate (7:3) as eluent: (a) UV 254 nm, (b) cerium(IV) sulfate, and (c) Dragendorff.

In accordance with the TLC results of ISM 7 crude extract, the sample underwent column chromatography fractionation using eluents comprising n-Hexane (100%), n-Hexane:ethyl acetate (9:1), n-Hexane:ethyl acetate (7:3), and culminating in methanol. The ISM 7 sample column chromatography process uses silica gel as a stationary phase, which is first hydrated with n-Hexane. The sample is eluted slowly using some type of predetermined eluent gradient.

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A total of 14 fractions have been obtained. All fractions were eluted on a TLC plate with the eluent n-Hexane 9:1 ethyl acetate, then visualized under 254 nm UV light, reacted with cerium(IV) sulfate and Dragendorff reagent; the results are shown in Table 4. The pattern of compound components produced tended to decrease, which indicates that the crude extract has been separated into upper, middle, and lower fractions. Fractions that have Rf values that tend to be the same can be combined into one fraction. The fractions that can be combined include fractions 1 and 2 (ISM7K1F1); fractions 3, 4, 5 (ISM7K1F2); fractions 6 and 7 (ISM7K1F3); fractions 8, 9, and 10 (ISM7K1F4); fraction 11 (ISM7K1F5); and fractions 12, 13, and 14 (ISM7K1F6). Samples (except ISM7K1F5 because it has a combined compound pattern between ISM7K1F4 and ISM7K1F6) were screened for antibacterial activity by testing 50 µL of a 2 mg/mL stock solution against *S. aureus* to determine the active fraction. The results of antibacterial screening of fraction samples are shown in Figure 6.

Table 4. TLC fractionation results of ISM 7 crude extract (fractions 1 – 14).

Eluent (n-Hexane:Ethyl acetate (9:1))		
UV 254	Cerium(IV) sulfate	*Dragendorff*
		
		

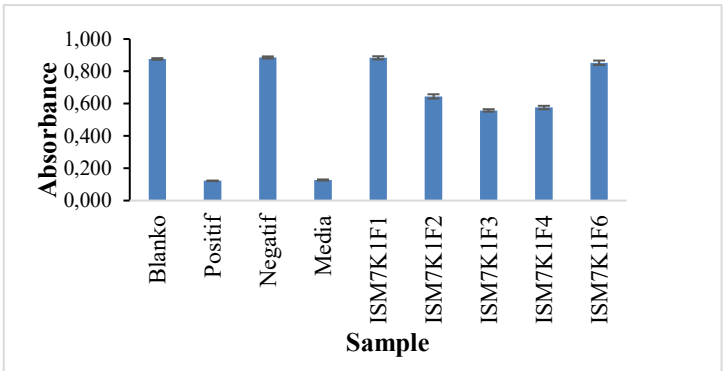


Figure 6. Growth of *S. aureus* after adding fraction extract.

The inhibitory power of the fractionated extract on bacterial growth at a concentration of 454 µg/mL was observed after 18 hours of incubation (Figure 6). All samples showed inhibition of bacterial growth except sample ISM7K1F1. Sample ISM7K1F3 showed the highest inhibitory power against *S. aureus*. TLC results in Table 5, the ISM7K1F3 sample produces a fairly strong orange stain after being reacted with Dragendorff's reagent compared to the other fractions, this indicates the presence of tertiary N groups (alkaloid components) in the sample, which may influence the antibacterial activity, because several studies reported that several alkaloid compounds have antibacterial potential (Ryu *et al.*, 2019; Shaala *et al.*, 2020; Wang *et al.*, 2020, 2021). ISM7K1F3

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physically has a white-yellow crystalline solid form so it must first be separated simply with n-Hexane solvent and produces white crystals with the code name ISM7K1F3 extract, as shown in Figure 7. The active fraction of ISM7K1F3 has a weight of 10 mg, which has been obtained. purified for further characterization of the compound.



(a) (b)

Figure 7. ISM7K1F3 sample (a) before separation and (b) after separation.

Compound Characterization

In accordance with the findings of the LC-MS/MS analysis, the Total Ion Chromatogram (TIC) graph for the ISM7K1F3 sample was obtained. The results of the LC-MS/MS analysis of the ISM7K1F3 sample demonstrated the presence of 17 peaks, of which a total of 8 were found to be present in high abundance, as illustrated in Figure 8. The chromatogram peaks were interpreted using MassLynx software to determine the approximate structure of the compound obtained based on the database on the ChemSpider website. The results of data interpretation are presented in Table 5.

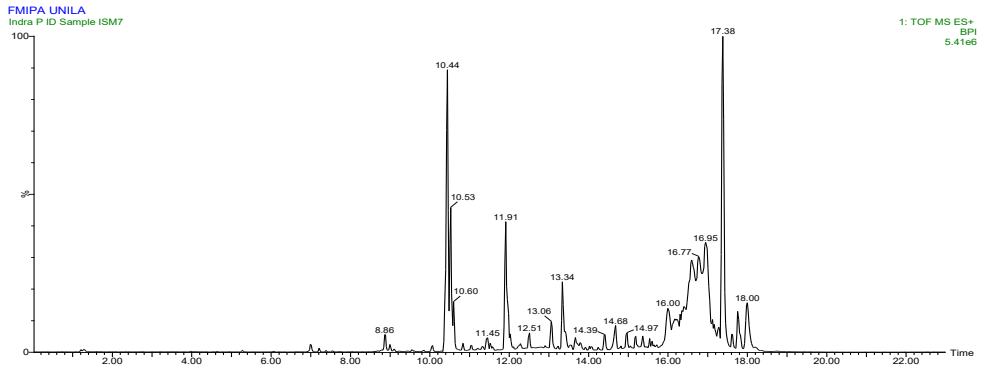


Figure 8. TIC of ISM7K1F3 sample.

Table 5. TIC chromatogram peak analysis of ISM7K1F3 sample.

No	t_R (min)	Formula	Calc. Mass	Fit Conf%	Compound Indication
1	10.44	$C_{16}H_{36}NO_2$	274.2746	99.97	*N*-[2-(2-Butoxyethoxy) ethyl]-*N*-butyl-1-butanamine
2	10.53	$C_{18}H_{40}NO_3$	318.3008	n/a	*N*-{2-[2-(2-Butoxyethoxy) ethoxy] ethyl}-*N*-butyl-1-butanamine
3	10.60	$C_{20}H_{44}NO_4$	362.3270	4.51	2-Butoxy-*N*-[2-(2-butoxyethoxy) ethyl]-*N*-(2-butoxyethyl) ethanamine
4	11.91	$C_{18}H_{40}NO_2$	302.3059	88.90	Tetradecyldiethanol- amine
5	13.34	$C_{20}H_{44}NO_2$	330.3372	n/a	*N, N*-Bis(2-ethoxyethyl)-1-dodecanamine
6	16.77	$C_{22}H_{45}N_6O_2$	425.3604	98.26	*N, N'*-1,2-Ethanediylbis (*N*-butyl-4-methyl-1-piperazine carboxamide)
7	16.95	$C_{26}H_{49}O_4$	425.3631	99.56	Bis(7-methyloctyl) Cyclohexane-1,2-dicarboxylate
8	17.38	$C_{22}H_{45}N_2O_4$	401.3379	0.70	*N*-[2-(1-hidroxybutoxy) ethyl]-*N*-[3-(morpholin-4-yl) propyl] nonanamide

The analysis of eight compound peaks with the highest abundance in the Total Ion Chromatogram (TIC) revealed that the compounds resulting from fragmentation were the main components, with the main peak at 17.38 minutes. The following is the chromatogram of the highest peak fragmentation at a retention time of 17.38 minutes in Figure 9.

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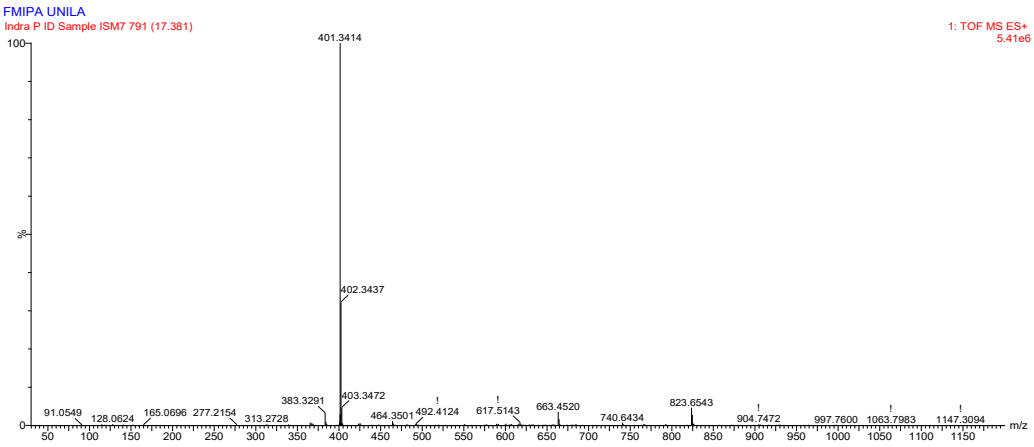


Figure 9. LC-MS/MS chromatogram of ISM7K1F3 sample retention time 17.38 minutes.

The LC-MS/MS chromatogram of sample ISM7K1F3 shows a base peak (m/z) of 401.3414, which is thought to indicate an alkaloid compound with the molecular formula $C_{22}H_{45}N_2O_4$ and based on the ChemSpider database, it is estimated to have the structural form of the compound as shown in Figure 10.

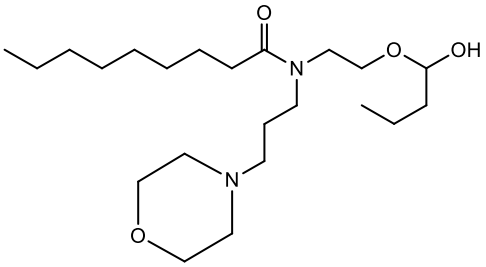


Figure 10. Estimated structure of the peak 17.38 compound for the ISM7K1F3 sample.

As illustrated in Figure 11, the results of data interpretation indicate that the estimated structure of the compound in the ISM7K1F3 sample is characterised by the fundamental framework of morpholine (tetrahydro-1,4-oxazine). This structure consists of a long-chain aliphatic C–C group, an O–H group, a C=O group, a C–O group, and a C–N group (tertiary). It is estimated that the structure of this compound contains a tertiary N group as a constituent. This is in accordance with the TLC data in Table 4 that in the ISM7K1F3 sample it is known that there is an orange spot at an Rf value of 0.56 after being reacted with Dragendorff's reagent which indicates the presence of a tertiary N group.

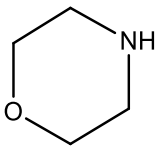


Figure 11. Basic structure of morpholine.

Based on the results of a literature review, morpholine derivative compounds are known to have antibacterial activity whether isolated from nature or synthesized. Xiao *et al.* (2017) reported the isolation of morpholine derivative compounds from the actinobacteria *Amycolatopsis* sp. and their subsequent demonstration of antibacterial activity against *S. aureus* at an MIC value of 4.8 µg/mL and Sanad *et al.* (2022) also reported that the synthesised morpholine derivative compound exhibited antibacterial activity against *S. aureus* at an MIC value of 7.71 µg/mL.

Fourier Transform Infra-Red Spectrophotometer (FTIR)

The results of the FT-IR analysis were used to obtain a spectrum, as illustrated in Figure 12. The FT-IR analysis data spectrum of the ISM7K1F sample indicates the presence of several types of functional group absorption, including a wave number of 1051.1 cm^{-1}, which is indicative of the existence of C–O bonds; a wave number of 1371.7 cm^{-1}, which is indicative of the absorption of the C–N group (tertiary); and a positive indication

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of the presence of a tertiary N group, as evidenced by an orange spot that appeared after reaction with Dragendorff. Furthermore, a wave number of 1461.1 cm^{-1} indicates the C–H bending alkane group, a wave number of 1714.6 cm-1 indicates the absorption of the C=O carbonyl group, a wave number of 2862.6 cm^{-1} and 2929.7 cm^{-1} indicate the presence of a C–H stretching alkane group, and the wave number 3414.2 cm^{-1} indicates the presence of an OH hydroxyl group (Nandiyanto *et al.*, 2019). These functional groups show a correlation that is in accordance with the estimated structure of the compound obtained from the LC-MS/MS analysis results, so it can be said that the results of the FT-IR analysis are in accordance with the functional groups contained in the compound structure proposed from the LC-MS analysis results.

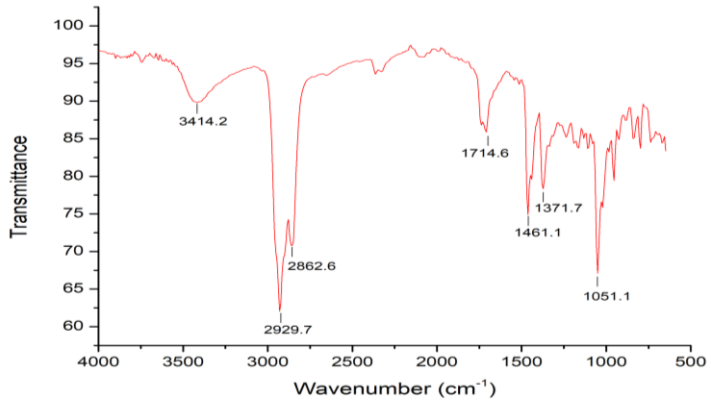


Figure 12. FT-IR spectrum of sample ISM7K1F3.

CONCLUSION

Seven actinomycetes isolates were obtained from mangrove sediments in Sriminosari, East Lampung, and were microscopically indicated as belonging to the genera *Streptomyces* and *Planomonospora*. The ISM7K1F3 fraction from isolate ISM7 showed the greatest inhibitory effect against *S. aureus* at 454 µg/mL. LC-MS/MS and FT-IR data suggested the presence of an alkaloid compound with a morpholine-like structural framework and O–H, C–H, C=O, C–N, and C–O functional groups. Further chemical characterization is required to confirm the proposed compound structure.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

SB: Conceptualization, Methodology, Investigation, Formal Analysis, Data Curation, Writing – Original Draft, Writing – Review and Editing, Supervision, Project Administration; AS: Methodology, Investigation, Resources, Supervision, Writing – Review and Editing; WAS: Methodology, Investigation, Resources, Validation, Writing – Review and Editing; YA: Investigation, Data Curation, Formal Analysis, Visualization, Writing – Review and Editing; IP: Investigation, Data Curation, Formal Analysis, Visualization, Writing – Review & Editing.

DECLARATION OF GENERATIVE AI

No declaration of generative AI use was provided by the authors.

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